IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

Yamamoto et al

Group Art Unit: 1636

Serial Number: 09/718,388

Examiner: Konstantina Katcheves

Filed: November 24, 2000

For: A METHOD FOR CULTURING CELL AND A CULTURE VESSEL

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner

Washington, D.C. 20231

Sir,

Nobutaka Yamamoto residing at c/o Menicon Co., Ltd., Central research laboratories, 1-10, Takamoridai 5-chome, Kasugai-shi, Aichi-ken, Japan duly deposes and says:

1. That he graduated from Department of Biology, Faculty of Science, Osaka University, Osaka, Japan and he received the degree of Master of Science from said University in the year 1997;

2. That he has been employed in the capacity since 1997 by Menicon Co., Ltd.;

3. That he has been engaged in research and development on cultured skin substitute and culture vessel.

4. That he is the present inventor, and has read and is familiar with the instant application for United States Letters Patent and the Office Action thereto mailed September 27, 2002;

5. That he has made experiments in order to prove that the method of present invention makes epidermal cells more effectively proliferate compared to a method for culturing epidermal cells

together with killed fibroblasts.

EXPERIMENTS

Each of the following experiments was carried out in triplicate.

Experiment 1 (Present Invention)

(i) The step of culturing fibroblasts

Established 3T3 mouse embryo fibroblasts were inoculated in a culture flask (culture surface: 25 cm^2) at 3×10^3 cells/cm^2 and incubated for 4 days in a CO_2 incubator (37 °C, 5 % CO_2) in Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum (DMEM + 10 % FBS).

After the step of culturing fibroblasts, a part of the culture surface on which fibroblasts attached was treated with a 0.25 % by weight of trypsin – EDTA solution, whereby the fibroblasts were separated and then collected. The number of the collected fibroblasts was counted and cell density of the culture surface was calculated. The result is shown in Table 1.

TABLE 1

	Number of collected fibroblasts (cells)	Cell density of the culture surface (cells/cm^2)
Flask 1	1.16×10^7	4.63×10^5
Flask 2	1.18×10^7	4.72×10^5
Flask 3	1.09×10^7	4.36×10^5
Average	1.14×10^7	4.57×10^5

(ii) The step of killing fibroblasts

After the incubation, culture supernatant in the culture flask was removed by aspiration, and the resulting culture vessel containing the fibroblasts was left to stand in a deep freezer at – 85 °C for 24 hours in order to kill the fibroblasts. The frozen culture vessel was thawed at room temperature, and then the culture vessel was left to stand in a deep freezer at -85°C for 2 hours. The frozen culture vessel was thawed again at room temperature.

(iii) The step of separating fibroblasts

The killed fibroblasts were separated from a surface of the culture vessel by rinsing the surface with 5 ml of Dulbecco's phosphate buffer (KCl, NaCl, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$) and removing the buffer from the culture vessel. The surface of the culture vessel is shown in Figure 1(a).

(iv) The step of inoculating epidermal cells

Epidermal cells were inoculated in the culture vessel at 1×10^4 cells/cm^2 and then cultured in Green's Medium supplemented with 3 % fatal bovine serum for 13 days in a CO_2 incubator (37 °C, 5 % CO_2). The surface of the culture vessel is shown in Figures 2(a) (day 2) and 3(a) (day 13).

Comparative Experiment 1

(i) The step of inoculating immortalized fibroblasts

Established 3T3 mouse embryo fibroblasts immortalized

with mitomycin C were inoculated at 3.0×10^4 cells/cm^2 in a culture flask, and cultured in DMEM + 10 % FBS for 19 hours in a CO_2 incubator (37°C, 5 % CO_2). The surface of the culture vessel is shown in Figure 1(b).

(ii) The step of inoculating epidermal cells

Epidermal cells were inoculated at 1×10^4 cells/cm^2 in the resulting culture flask in which the immortalized fibroblasts attached, and then incubated in Green's Medium supplemented with 3 % fatal bovine serum for 13 days in a CO_2 incubator (37 °C, 5 % CO_2). The surface of the culture vessel is shown in Figures 2(b) (day 2) and 3(b) (day 13).

Comparative Experiment 2

(i) The step of inoculating immortalized fibroblasts

Established 3T3 mouse embryo fibroblasts immortalized with mitomycin C were inoculated at 5.0×10^4 cells/cm^2 in a culture flask, and cultured in DMEM + 10 % FBS for 19 hours in a CO_2 incubator (37°C, 5 % CO_2). The surface of the culture vessel is shown in Figure 1(c).

(ii) The step of inoculating epidermal cells

Epidermal cells were inoculated at 1×10^4 cells/cm^2 in the resulting culture flask in which the immortalized fibroblasts attached, and then incubated in Green's Medium supplemented with 3 % fatal bovine serum for 13 days in a CO_2 incubator (37 °C, 5 % CO_2). The

surface of the culture vessel is shown in Figures 2(c) (day 2) and 3(c) (day 13).

Conclusion

As a result of Experiment 1 and Comparative Experiments 1 and 2, it was understood that the colonies prepared in Experiment 1 were larger than those prepared in Comparative Experiments 1 and 2 (see Figures 2(a), 2(b) and 2(c)). Thirteen days after inoculation of epidermal cells, it was observed that the epidermal cells completely covered the culture surface in Experiment 1, but not in Comparative Experiments 1 and 2. Furthermore, epidermal cells proliferate more in Experiment 1 than Comparative Experiments 1 and 2 (see Figure 4).

Therefore, it is proved that the method of present invention makes epidermal cells more effectively proliferate compared to a method for culturing epidermal cells together with killed fibroblasts.

FIG. 1 (a)

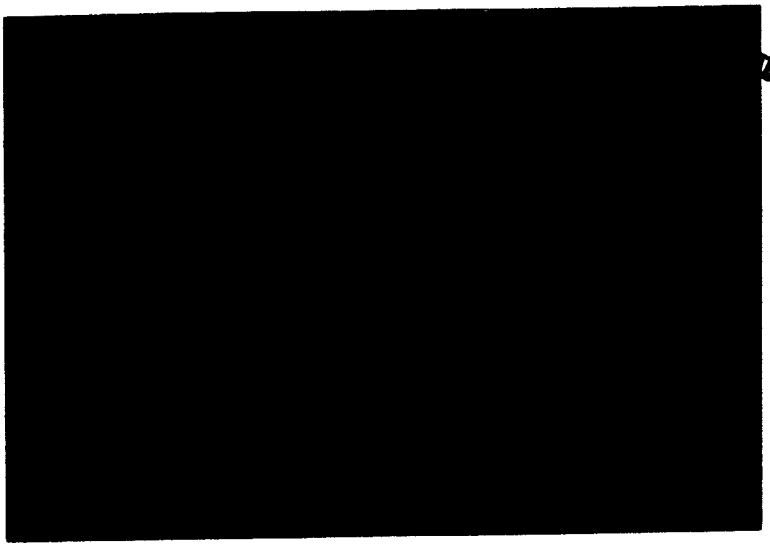


FIG. 1 (b)

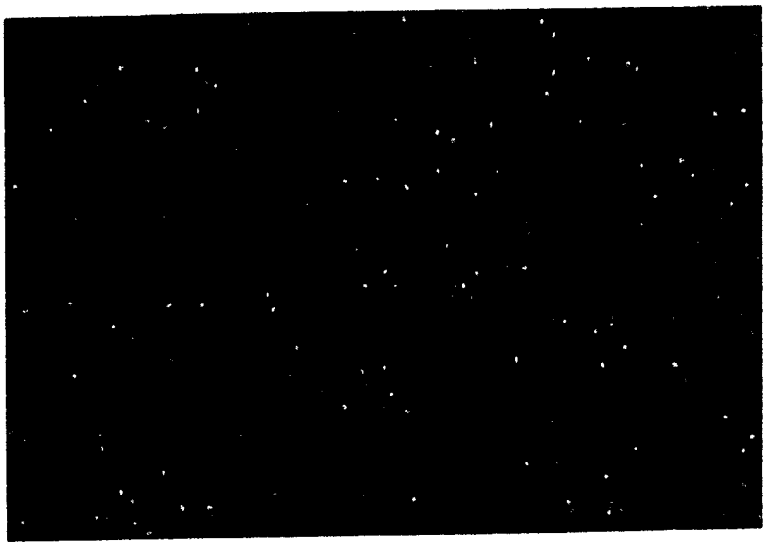


FIG. 1 (c)

FIG. 2(a)

FIG. 2(b)

FIG. 2(c)

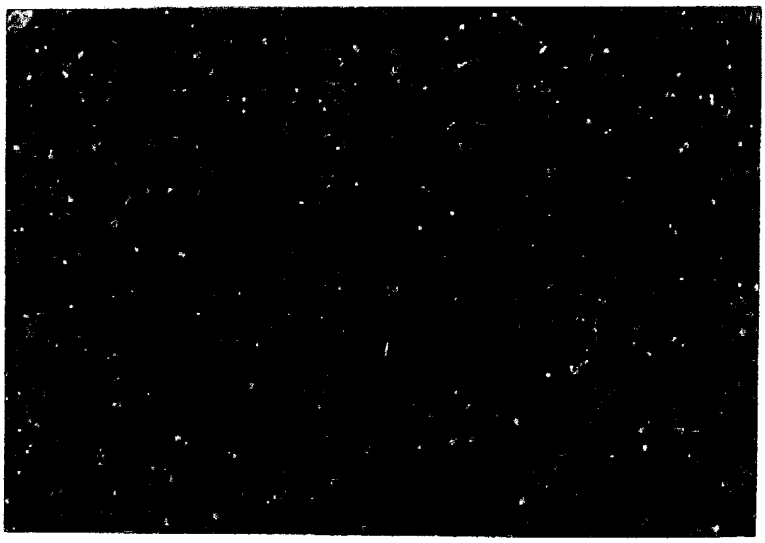


FIG. 3(a)

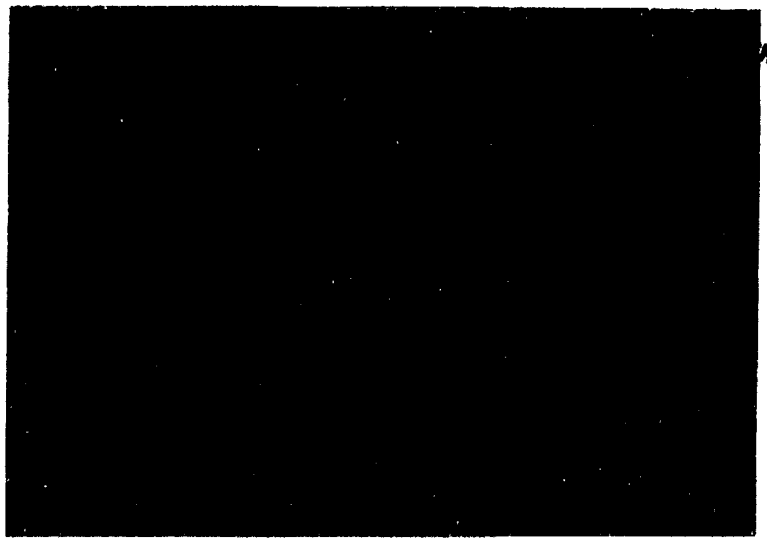
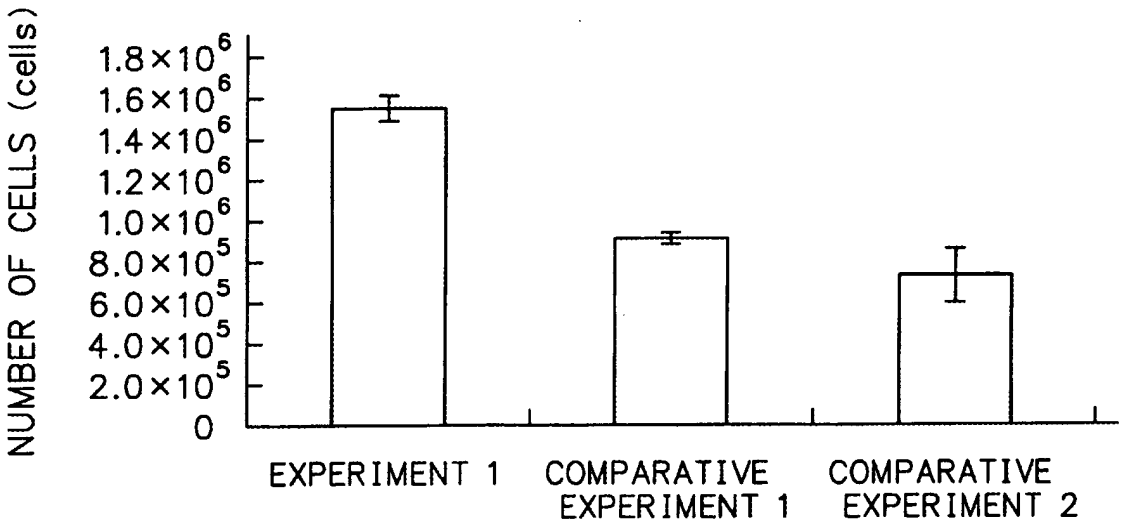


FIG. 3(b)

FIG. 3(c)

FIG. 4



The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 5th day of September, 2003

by Nobutaka Yamamoto

Nobutaka Yamamoto

We, the undersigned witnesses, hereby acknowledge that Nobutaka Yamamoto is personally known to us and did execute the foregoing Declaration in our presence on:

Date: September 8, 2003 Witness Naoka Yamamoto

Naoka Yamamoto

Date: September 8, 2003 Witness James Jester

James Jester, Ph.D